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Bursaphelenchus fungivorus from *Pinus pinaster* bark in Portugal

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Summary

Bursaphelenchus fungivorus is reported for the first time in Portugal, identified as associated with *Pinus pinaster* bark and characterized on the basis of morphological and morphometrical characters for this species. Species identification was confirmed using restriction fragment length polymorphism analysis and sequencing of the internal transcribed spacer (ITS) regions of ribosomal DNA. Intraisolate genetic variability was detected among ITS sequences of the Portuguese *B. fungivorus* isolate. Phylogenetic analysis, obtained from multiple sequence alignment between ITS sequences of *Bursaphelenchus* species, revealed that the Portuguese *B. fungivorus* isolate clusters with other *B. fungivorus* isolates, forming a separate group close to *B. seani* highlighting a molecular proximity of these two species.

1 Introduction

The genus *Bursaphelenchus* was established by Fuchs in 1937 and includes nematodes that are associated with insects and dead or dying trees, mainly conifers. This genus comprises approximately 100 species, most of them wood-inhabiting and fungal-feeding nematodes that inhabit several conifer trees (Hunt 2008; Vicente et al. 2012). Most *Bursaphelenchus* spp. are mycophagous and are transmitted to dead or dying trees during oviposition by insect vectors or to healthy trees during maturation feeding by the vector. Most of these vectors are beetles belonging to the Scolytidae, Cerambycidae, Curculionidae and Buprestidae families (Ryss et al. 2005). In Portugal, several species (*B. antoniae*, *B. hellenicus*, *B. leoni*, *B. mucronatus*, *B. minutus*, *B. pinasteri*, *B. pinophilus*, *B. sexdentati*, *B. tusciae* and *B. xylophilus*) have been described associated with maritime pine, *Pinus pinaster*, wood and insects (Penas et al. 2004, 2006a,b; Ryss et al. 2005; Fonseca et al. 2012; Vieira and Mota 2013).

Bursaphelenchus fungivorus is a mycophagous species and was first described on rotting flower buds of a greenhouse gardenia (*Gardenia* sp.) from North Wales, infected by the fungus *Botrytis cinerea* (Franklin and Hooper 1962) and later in a growing medium containing bark for *Pelargonium* plants in a greenhouse in Germany (Braasch et al. 1999a). Owing to phytosanitary measures imposed on coniferous bark and wood trade, it has been also found in coniferous bark imported from the Czech Republic and Russia to Germany (Braasch et al. 2002), in wood chips and sawdust from sawmills in Germany (Schönfeld et al. 2008) and in Chinese packaging wood of *Callitris columellaris* imported to Italy (Ambrogioni et al. 2003). *Bursaphelenchus fungivorus* was also reported on *Pinus* spp. and associated with *Orthotomicus erosus* in Spain (Arias et al. 2004, 2005). Moreover, it was also detected in Brazil, extracted from the fibrous husk (mesocarp) of coconut (*Cocos nucifera*) (Oliveira et al. 2011). Regarding the pathogenicity of this species to *Pinus* spp., it was described as pathogen to 3-year-old *P. sylvestris* seedlings in experimental climate chamber studies (Braasch et al. 1999b). However, its presence in *P. pinaster* and other *Pinus* spp. associated with any wilting symptoms has not been reported in the field (Arias et al. 2004, 2005).

In this study, *B. fungivorus* is reported for the first time in Portugal associated with *P. pinaster* bark and characterized morphologically, morphometrically and molecularly by internal transcribed spacer (ITS) regions of rDNA restriction fragment length polymorphism (RFLP) analysis and sequencing.

2 Materials and methods

2.1 Nematodes extraction and culture establishment

Nematodes of the genus *Bursaphelenchus*, extracted from maritime pine (*P. pinaster*) bark by the tray method (Whitehead and Hemming 1965), were handpicked and transferred to the fungus *Botrytis cinerea* grown, at 25°C, on malt extract agar, in order to obtain a culture. After 1 month, the nematodes were washed from the fungus with sterilized distilled water and collected for morphobiometrical and molecular studies.

2.2 Morphobiometrical characterization

Twenty females and 20 males were killed by heat in a drop of water on a cavity glass slide, mounted in water, viewed, photographed and measured immediately. Photographs were taken with a LeitzDialux 20 bright field light microscope (Ernst LeitzLtd., Midland, Ontario, Canada) using a LeicaDFC 450 digital camera (Leica, Heerbrugg, Switzerland). Measurements were performed with the LEICA MICROSYSTEM LAS INTERACTIVE MEASUREMENT SOFTWARE Version 4.0.0.

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2.3 DNA extraction and amplification of ITS regions

DNA from 10 nematodes was extracted with the DNeasy® Blood and Tissue Mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The ITS rDNA regions containing partial 18S and 28S and complete ITS1, 5.8S and ITS2 sequences were amplified using 50 ng extracted DNA and 1 U Dream Taq DNA polymerase (Fermentas, Hanover, NH, USA) in 1X Dream Taq buffer, 0.2 mM each dNTP and 1 μM primers 18SF 5′-CGTAACAAGGTAGCTGTAG-3′ (Ferris et al. 1993) and 28SR 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain 1993). All reactions were carried out in a Thermal Cycler (Bio-Rad, Madrid, Spain) with an initial denaturation step of 95°C for 2.5 min followed by 40 reaction cycles of 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The resulting amplification product was used for RFLP analysis and sequencing.

2.4 Restriction fragment length polymorphism analysis

The RFLP analysis of amplified ITS rDNA PCR product was performed using *Afa*I, *Alu*I, *Hae*III, *Hinf*I and *Msp*I endonucleases (Amersham Biosciences, Buckinghamshire, UK), according to the manufacturer's instructions. PCR and restriction products were separated by electrophoresis on 1.5% agarose gel, and fragment sizes were estimated using Hyper Ladder II (Bioline, Luckenwalde, Germany).

2.5 Sequencing and phylogenetic analysis

For sequencing, the amplified ITS rDNA product was first purified with the Qiaquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions, and then directly sequenced in both strands in an Automatic Sequencer 3730xl under BigDye™ terminator cycling conditions at Macrogen Company (Seoul, Korea), using the same primers as in PCR and IKF1 and IKF2 primers (Iwahori et al. 1998). To improve sequencing results, the purified PCR product was further cloned and three clones completely sequenced in both strands with the universal primers M13Fwd and M13Rev, by Macrogen Company. Sequences analyses were carried out using BioEdit (Hall 1999) and nucleotide diversity (± standard deviation) was estimated in DnaSP 5.10 (Librado and Rozas 2009). The beginning and end of the ITS regions were delineated based on the NCBI databases. Homologous sequences in the databases were searched using BLAST (Altschul et al. 1997) and used for phylogenetic analysis. A phylogenetic tree was constructed in MEGA 5.1 (Tamura et al. 2007) by the neighbour-joining method (Saitou and Nei 1987) with 1000 replications of bootstrap and Jukes-Cantor as substitution model, using the ITS sequences alignment of the obtained ITS sequence and the homologous ones found in the databases.

3 Results

3.1 Morphobiometrical characterization

Females and males presented the main morphological diagnostic characters of *B. fungivorus*: cephalic region offset by a constriction, with six lips; stylet with weakly developed basal knobs; median bulb well developed; vulva without flap and a long tail ventrally bent in the females; and tail ventrally curved with compact spicules without cucullus in the males (Fig. 1). Most of the morphometric data of males and females (Table 1) were similar to those found for other *B. fungivorus* isolates. The Portuguese isolate was then designated as BfungPt1.

Table 1. Morphometrics of females and males of *Bursaphelenchus fungivorus* (BfungPt1).

Linear (μm)	Females (n = 20)	Males (n = 20)
Body length (L)	682.9 ± 119 (468.2–983.9)	617.8 ± 61.9 (543.8–788.3)
Stylet length	13.9 ± 0.9 (12.1–15.1)	13.9 ± 0.8 (12.4–15.9)
Greatest body width (GBW)	31.3 ± 5.9 (23.1–37.4)	26.5 ± 4.2 (20.3–37.6)
Distance from anterior end to end of median bulb (DAEEMB)	63.0 ± 6.7 (50.8–73.1)	62.7 ± 6.9 (47.9–73.7)
Distance from anterior end to vulva (DAEV)	488.5 ± 96.7 (327.3–732.7)	–
Distance from vulva to anus	144.7 ± 28.9 (223.5–100.6)	–
Tail length (TL)	51.5 ± 9.0 (39.3–68.7)	35.2 ± 3.6 (29.9–43.3)
Body width at anus (BWA)	13.1 ± 2.2 (9.2–17.2)	16.7 ± 2.1 (13.8–20.8)
Spicule length	–	14.2 ± 1.0 (12.5–16.3)
Ratio		
a = L/GBW	21.8 ± 2.2 (18.1–26.3)	23.6 ± 2.5 (19.0–28.0)
b_1 = L/DAEEMB	11.0 ± 2.5 (7.5–15.8)	10.0 ± 1.4 (7.8–13.1)
c = L/TL	13.6 ± 3.5 (9.4–24.0)	17.7 ± 2.2 (13.2–22.4)
c' = TL/BWA	4.0 ± 0.8 (3.1–5.2)	2.1 ± 0.2 (1.9–2.5)
Percentage		
V = DAEV × 100/L	71.3 ± 2.5 (66.7–75.3)	

Values are mean ± SD.
Values in parentheses indicate the minimum and maximum.

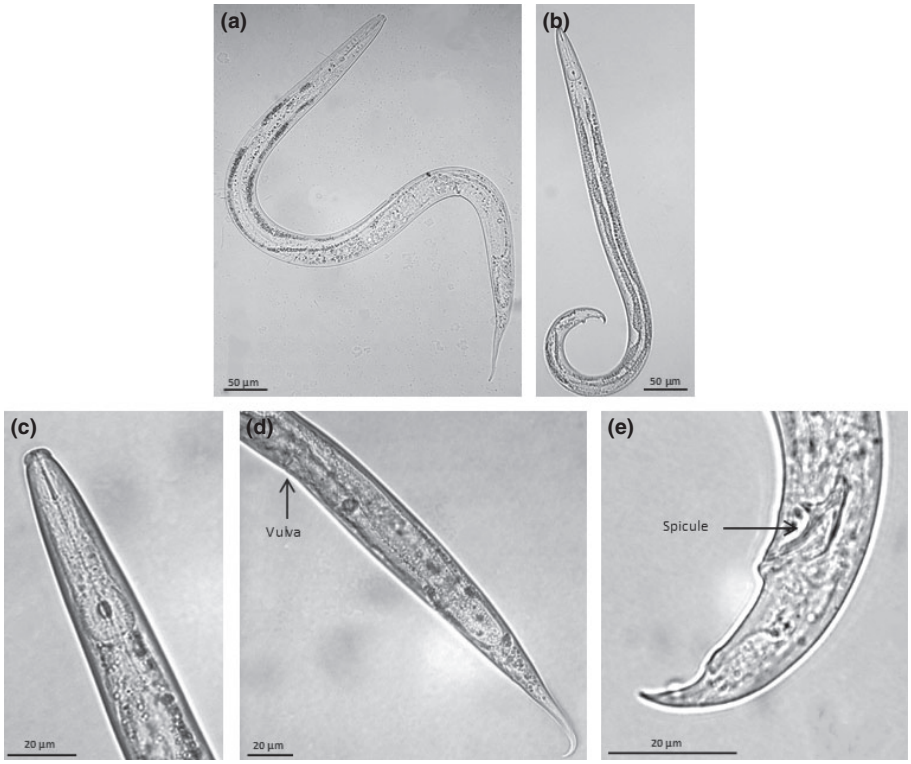

Fig. 1. Light microscope photographs of *Bursaphelenchus fungivorus* (BfungPt1). (a) female, (b) male, (c) anterior end, (d) female vulvar region and tail (e): male tail.

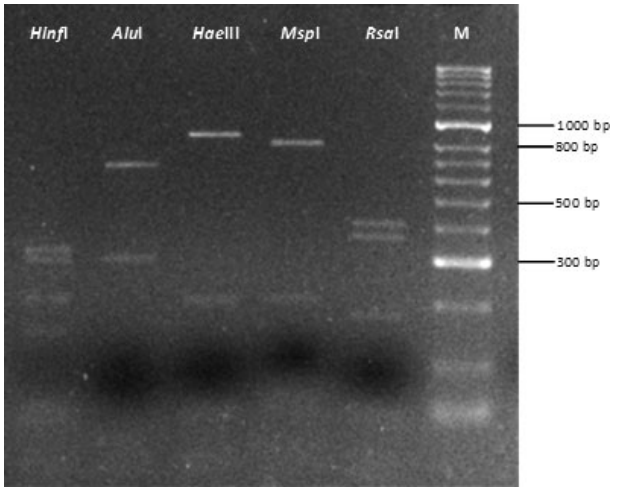

Fig. 2. ITS-RFLP patterns of a *Bursaphelenchus fungivorus* (BfungPt1) with five restriction endonucleases. M: DNA size marker (Hyper Ladder II, Bioline).

3.2 Molecular identification

The amplification of BfungPt1 ITS rDNA regions yielded a single product of approximately 1050 bp, and the restriction enzyme patterns obtained after *Afa*I, *Alu*I, *Hae*III, *Hinf*I and *Msp*I digestion (Fig. 2) were in agreement with those reported for *B. fungivorus* species (Arias et al. 2005; Burgermeister et al. 2009; Oliveira et al. 2011).

Sequencing chromatograms obtained directly from purified PCR product had some regions with mixed signal, indicating some gaps/insertion in the sequences, revealing intraisolate molecular variability. Three clones were then sequenced and the ITS sequences submitted to NCBI databases under the accession numbers KF241745, KF241746 and KF241747. Some differences were detected among the three clones varying in size from 998 to 1018 bp (excluding the primers sequences) with an estimated nucleotide diversity of 0.00802 ± 0.00292.

The phylogenetic analysis, from multiple sequence alignment between ITS sequences of BfungPt1 and of other *Bursaphelenchus* species, revealed that BfungPt1 clusters together with a Chinese *B. fungivorus* isolate (HQ402559) and a German isolate (AM179516) forming a separate group close to a *B. seani* isolate (AM15775) (Fig. 3).

4 Discussion

The morphology of the Portuguese *B. fungivorus* isolate agrees with the original description (Franklin and Hooper 1962) and with the morphological characteristics indicated by other authors for this species (Braasch 2001; Arias et al. 2004, 2005; Oliveira et al. 2011). Most of the morphometric data of the Portuguese isolate (Table 1) are within the range of other *B. fungivorus* isolates (Franklin and Hooper 1962; Braasch 2001; Arias et al. 2004, 2005; Oliveira et al. 2011) apart of the *a* ratio (L/GBW), which is smaller.

The molecular data in this study clearly identify BfungPt1 as *B. fungivorus*. However, some intraisolate genetic variability was found among the determined ITS sequences of BfungPt1 isolate, which is in agreement with the intraisolate molecular variability described for *B. xylophilus* (Cardoso et al. 2012), *Meloidogyne* species (Hugall et al. 1999) or non-phytoparasitic nematodes (Blouin 2002).

The phylogenetic analysis, using the ITS regions, revealed that the Portuguese, Chinese and German isolates of *B. fungivorus* group with a *B. seani* isolate. This result is in accordance with those obtained with ITS1, 5.8S and ITS2 sequences, in

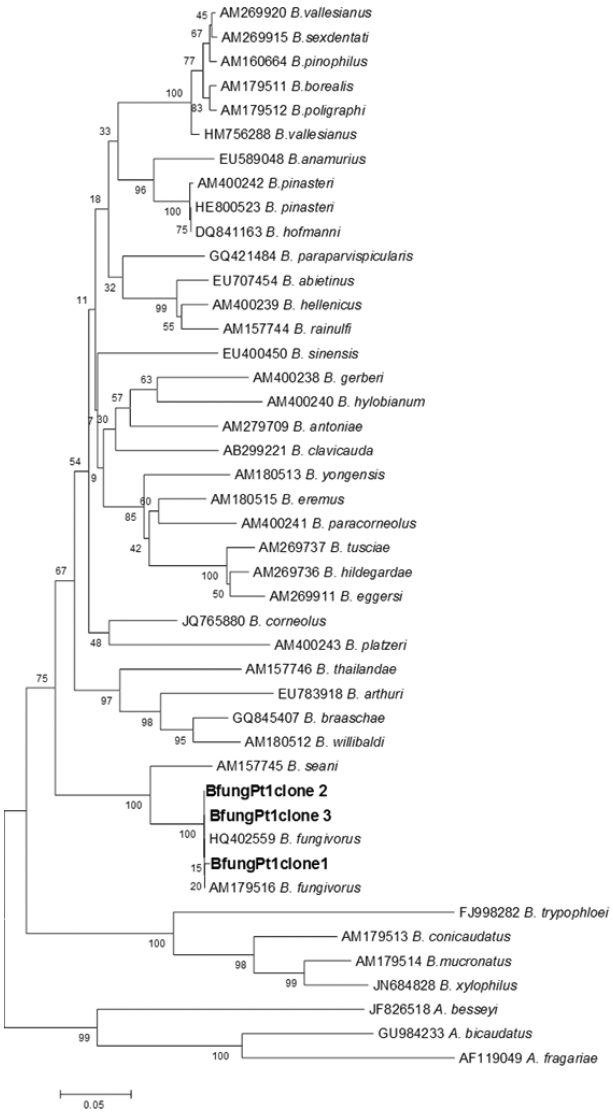

Fig. 3. Phylogenetic tree generated by neighbour-joining method using the multiple sequence alignment between internal transcribed spacer (ITS) sequences of the three clones of the Portuguese *Bursaphelenchus fungivorus* isolate (BfungPt1) and of other *Bursaphelenchus* species present in NCBI databases. The ITS sequences of the species *Aphelenchoides besseyi*, *A. bicaudatus* and *A. fragariae* were used as out-groups. Bootstrap values are shown at branch points, and scale bar indicates 0.05 substitutions per site.

separate and in combined sequence analysis, and with small subunit (SSU) ribosomal RNA, large subunit (LSU) ribosomal RNA and mitochondrial cytochrome oxidase subunit I (mtCOI) sequence data, revealing a molecular proximity of these two species (Ye et al. 2007; Metge et al. 2008). The SSU and LSU partial gene sequences of a Brazilian *B. fungivorus* isolate have also been previously described and revealed to be 100% identical to the German isolate and 99% identical to a *B. seani* isolate (Oliveira et al. 2011). This DNA sequence homology between *B. fungivorus* and *B. seani* species is consistent with the groups previously defined by morphological features, according to which both species belong to the *fungivorus* group (Braasch 2009).

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